Exercise 1, Molecular identification of clinical strains

Introduction

Microbial diseases are quite heterogeneous; they can affect any organ and thus can produce many different symptoms. Hence they are related only by their common cause: pathogenic microorganisms. Indeed, this is the only large group of disease of which the primary cause can in most cases be identified. Identification of the causative agents in microbial diseases is crucial in order to prescribe efficient treatment procedures, and in order to determine the distribution and frequency (epidemiology) of the diseases, which provide a basis for disease control. Identification of the microorganisms has traditionally been carried out on the basis of phenotypic traits observed by microscopy, staining, and biochemical tests, but today identification of microbes additionally depends to a large extent on ribosomal RNA (rRNA) sequences. Methods which use rRNA for identification include whole-cell fluorescent in situ hybridization (FISH), ribotyping such as 16S rRNA restriction fragment length polymorphism (RFLP), and rRNA sequencing followed by comparison to ribosomal databases. These methods are all accurate and fast since they can be carried out without cultivation.

Biochemical testing is in the clinic carried out by seeding on various selective media but this only provide a coarse subdivision into classes of organisms, information which in most cases is sufficient to enable the physicians to provide adequate care for the patient. A modern development of this method (BIOLOG ®) use the same principle but tests many carbon sources at a time in microtiter trays and in combination with a large database, it is usually possible to precisely determine the species tested.

FISH is used extensively for identification of specific organisms in clinical settings. The occurrence of highly conserved, as well as variable, regions in the rRNA makes it possible to design probes for identification of the microorganisms at any chosen phylogenetic level. FISH comprise a chemical fixation of the microbial cells followed by hybridization with specific fluorescence-labeled oligonucleotide probes (15-25 nucleotides) targeted against specific sequences in the 16S or 23S rRNA. The fluorescent cells can then be visualized by epifluorescense or laser scanning microscopy.

16S rRNA RFLP analysis is carried out on PCR-amplified rDNA from either a pure culture or a mixed-species community. In the present exercise we use PCR primers targeting conserved regions, so DNA from all species will give rise to a PCR product. Between the conserved regions each species contain unique sequences in the rRNA, and therefore the abundance and types of restriction sites in the PCR fragments will vary. Hence, digestion of the PCR products with restriction enzymes followed by agarose gel electrophoresis will yield different restriction fragment length patterns. These RFLP patterns are used as fingerprints for identification of the species by comparison with known standards.

Species identification can ultimately be performed by sequencing of rDNA PCR-amplified fragments and comparison to ribosomal databases.

Aims

In the present exercise you will get cultures with bacteria which could have been isolated from a patient suffering from a microbial disease. The aim of the exercise is to identify the bacteria by the use of basic selection media, BIOLOG, 16S rRNA FISH, RFLP, and sequencing.

Experimental procedures (Please refer to the course time table to see which days to do what)

Day 1 (8 in the timetable)

(1A, 1B, 1C, 1D) Inoculation in liquid medium and onto fresh plates.

Pick single colonies from the plates with the unknown clinical isolates and inoculate each of the two strains in 10 mL LB medium and shake o.n. at 37°C. Label the tubes with your team number and "ex1-1" or "ex1-2"

Streak out a colony on a Blood-agar (BA) plate and inoculate at 30°C until day 3.

Get one additional unknown strain by using a sterile cotton swap and sample a part of your body (ear, nose, armpit etc) or some part of the lab which is frequently touched, e.g. a door handle, water tap, computer keyboard etc. Spread the sampled bacteria by rotating the cotton tip in a small area of a BA plate and spread to single colonies using sterile single use inoculation loops.

Note: NEVER EVER put a cotton swap or inoculation loop back in the plastic bag it came from, even if it has not been used. The same rule applies to eppendorf tubes and pipette tips.

Label the three plates with team number and "ex1-1", "ex1-2" and "ex1-3" respectively.

Day 2 (9 in the timetable)

(1B) Preparation of chromosomal DNA from each of the 2 clinical isolates:

Materials
10 mL TNE buffer
2 mL TNEX
200 µL Lysozyme (5 mg/ml)
50 µL Proteinase K (20 mg/ml)
200 µL 5M NaCl
EtOH
100mL dH_2O

1. Harvest 500 µl culture : Spin down cells (5 min at 7000 g) and decant.
2. Wash with 1 ml TNE : Resuspend in 1 ml TNE; vortex; spin down cells (5 min at 7000 g); decant.
3. Resuspend pellet in 270 µl TNEX.
4. Add 30 µl of a freshly prepared lysozyme solution (5 mg/ml in H_2O).
5. Add 7.5 µl Proteinase K solution (20 mg/ml in H_2O).
6. Incubate for 90 min at 37°C and then at 65°C for another 90 min.
7. Add 15 µl 5 M NaCl and mix gently by inverting the tube.
8. Add gently 1 ml 96% EtOH. After 2-3 min mix gently and incubate 15 min at -20°C.
9. Spin at 15000 g for 10 min at 4°C.
10. Wash pellet with 1 ml ice cold 96% EtOH. Be careful! Don't loose the DNA!
11. Spin at 15000 g for 10 min at 4°C.
12. Remove remaining EtOH by pipetting followed by incubation at 37°C for 3-5 min with the lid open (do not over dry pellet).
13. Add 500 µl dH_2O and mix gently by inverting the tube. Store at 20°C o.n. to dissolve the DNA; then at -20°C.

Don't vortex chromosomal DNA or it will shear.

(1A) Fixation of bacteria from each of the 2 isolates (LB tubes from day 1)

Materials
Fixative
PBS buffer
0,1% Nonidet P-40
2x storage buffer
EtOH 96%

Warning - Fixative (paraformaldehyde) is carcinogenic. Work with nitril-gloves. Use the hood.

1. Spin down cells (2 ml o.n. culture) (8 min at 7000 g).
2. Remove 1750 µl of the supernatant; resuspend cells in the remaining 250 µl.
3. Add 750 µl of fixative, vortex for 1 min and keep at 4°C for 15 min.
4. Spin down cells (5 min at 7000 g), decant supernatant carefully in the fixative waste container in the hood.
5. Resuspend cells in 900 µl of PBS buffer, vortex 1 min.
6. Add 100 µl 0.1% Nonidet P-40, vortex for 1 min.
7. Harvest cells by centrifugation (5 min at 7000 g); decant supernatant carefully.
8. Resuspend cells in 25 µl of 2 x storage buffer, vortex 1 min.
9. Add 25 µl 96% EtOH, vortex 1 min.
10. Store samples at –20°C until use.

(1A) Fluorescent in situ hybridization

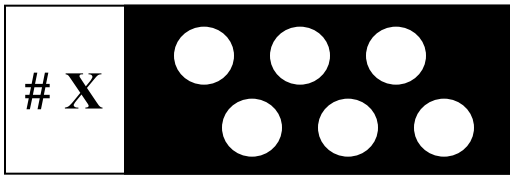
Materials (shared among all groups)
EtOH 96%, 80% og 50%
Hybridization solution 30%FA
2x100mL Wash I
2x100mL Wash II
dH_2O

Warning - Use the hood for the entire procedure.

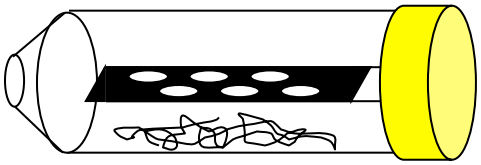
Probes for hybridization of the two clinical isolates:

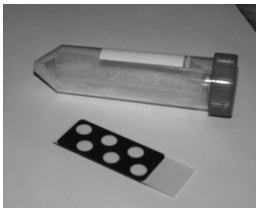
Probe	**Sequence 5'→3'**	**Label**
Gram negative, β-proteobacteria	GCC TTC CCA CTT CGT TT	CY3
Gram negative, γ-proteobacteria	GCC TTC CCA CAT CGT TT	CY3
Bacteria	GCT GCC TCC CGT AGG AGT	FITC

The FISH analysis is performed on a 6-well heavy Teflon ® slide coated with poly-L-lysin. Write your name or group number in the transparent field on the slide using a pencil.



1. Two µl of fixed cells are distributed homogeneously over the surface of a well on a coated teflon slide with the help of a pipette tip (be careful, don't scratch the poly-L-lysin coating). One isolate is put in 3 wells.
2. After air-drying the immobilized cells are dehydrated by transferring the slide successively into three 100 ml coplin jars containing ethanol solutions of 50%, 80% and 96%. Dip the slide 3 min (not more!) in each coplin jar.
3. After drying the slides at room temperature, 20 µl of hybridization solution (Wash I containing ca. 25 ng/µl of a probe: the beta-specific, the gamma-specific or the general eubacterial probe) is applied to each well (Each strain is located in 3 wells: one per probe). The slides are incubated at 37°C in the dark for 3 hours in a formamide-saturated atmosphere (in a sealed large test tube containing tissue paper wetted with the hybridization buffer (= wash I)).





4. To remove unspecific bound probe, two washing steps are performed as follows: Rinse slides in dH_2O, transfer them quickly to a coplin jar with prewarmed (37°C) washing solution I, and incubate at 37°C for 20 min. Transfer the slides to 100 ml of washing solution II (37°C) and incubate for 15 min at 37°C. Rinse the slides with dH_2O and air dry. Keep the slides in the dark until epifluorescence microscopy is performed to avoid bleaching.

Warning – Hybridization buffer (formamide) is carcinogenic. Work with nitril-gloves (blue). Use the hood.

Day 3 (10 in the timetable)

(1D) AB-test and Blue agar test

To test the unknown strains for possible resistance to antibiotics we will spread them onto BA plates and apply Rosco AB-tablets on the surface. Any resistance will result in a clearing zone, which size indicates how resistant the strain is. In addition we make a screening on a blue plate to identify gram-positive strains, and to check whether the strain can ferment lactose. See appendix for details.

Since you have not restreaked the isolate from your body (plate ex1-3), you will need to do that now. Pick a single colony and streak to single colonies on a new BA plate. Label the plate with team number and "ex1-4" and incubate at 37C o.n.

Dry three BA plates and prepare them for the AB test. We will test for 4 different antibiotics, ampicillin, gentamycin, vancomycin and erythromycin (polymyxins). Since Vancomycin diffuse poorly into the plates we need to pre-inoculate the pates with this Rosco-tab prior to spreading the test organisms. On the back of the three plates write the letters A, G, V, and P with a marker. Using a sterile pincet, place a Van test tablet on the surface on the BA plate on top of the letter "V". Leave the tab on the agar surface for 2 hours. Clean a work area on your table with 70% EtOH before proceeding and observe sterile technique when handling the plates! Then remove the tab by gently tapping the plate upside down on the clean area of your bench. Dispose of the tablet and store the plates in a plastic bag o.n. on the bench.

(1A) Epifluorescence microscopy of FISH labeled clinical isolates:

The principle of epifluorescence microscopy is based on the fact that some molecules, the so-called fluorochromes, emit part of the light absorbed by them as longer waves.

Three components are needed in an epifluorescence microscope:

1. A strong source of light, which emits mainly short lightwaves. Mercury or Xenon high-pressure lamps have proven useful.
2. An excitation filter: This filter helps to shut off all radiation other than the one that activates the specific fluochrome. It is placed behind the light source within the light cone.
3. The emission filter: This filter is brought into the light cone between objective and eyepiece. It lets through only longer wavelengths caused by emission from the fluorochrome.

In the present exercise we use oligo-nucleotides labeled with FITC or CY3. The FITC fluorochrome has an excitation peak wavelength of 490 nm, and an emission peak wavelength of 520 nm (i.e. green light). The CY3 fluorochrome has an excitation peak wavelength of 552 nm, and an emission peak wavelength of 565 nm (i.e. red light). Each of the wells with the hybridized cells is analyzed using phase contrast microscopy, and epiflurescence microscopy with the FITC

optical filter or the CY3 optical filter.

(1B) 16 S rDNA PCR amplification on chromosomal DNA from each of the 2 isolates.

Materials
Sterile MilliQ water
10xPCR buffer
dNTP 10mM
9F primer, 5'-GAGTTTGATCCTGGCTCAG-3' (100µM)
1512R primer, 5'-ACGGCTACCTTGTTACGACTT-3' (100µM)
DMSO
Taq-polymerase

Everything needs to be on ice until the PCR is running. The 16S rDNA from the 2 isolates is amplified by the use of PCR with the primers 9f (5'-GAGTTTGATCCTGGCTCAG-3') and 1512r (5'-ACGGCTACCTTGTTACGACTT-3'). Prepare a PCR master mix with 4 x each of the components written below, except the DNA template and Taq-polymerase. Pipette 48,5 µl of the master mix in 3 PCR tubes and add 1 µl chromosomal DNA solution to 2 of the tubes; one tube for each species! The third tube is a negative control without DNA. The Taq-polymerase will be added by the teacher just before the run. The master mix is made in excess to cover losses during pipetting. The final content of each of the 3 samples should be:

34.5 µl sterile MilliQ water
5 µl PCR buffer (*tag* buffer with $(NH_4)_2SO_4$, no $MgCl_2$)
2 µl dNTP
1 µl 9F primer
1 µl 1512R primer
1 µl DMSO
4 µl $MgCl_2$
1 µl DNA template (not in the case of the negative control!)
and
0.5 µl Taq-polymerase

Important – Keep everything on ice until the PCR is running and work sterile!

The PCR is done with the following temperature profile: 95°C 5 min; 30 cycles of: 94°C 30 sec, 53°C 30 sec, 72°C 90 sec, 72 °C 5 min; thereafter 4°C until storage at -20°C.

(1B) Sequencing of the PCR product
Give the remainder of the PCR product to the teacher for off-site sequencing – Sequences will be returned in week 3.

Day 4 (11 in the timetable)

(1C) RFLP analysis: Restriction cutting of PCR products

Materials

Restrictionenzymes:

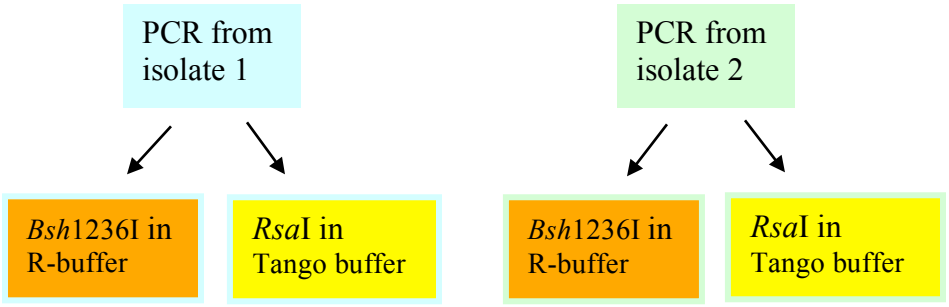
*Bsh*1236I

*Rsa*I

10uL R-buffer

10uL Tango buffer

The products from the PCR reactions are restriction enzyme digested with *Bsh*1236I in R-buffer and with *Rsa*I in Tango buffer. In both cases use: 5 µl dH_2O, 8 µl PCR product, 1.5 µl buffer (both buffers are 10 x concentrated), and 0.5 µl of restriction enzyme solution. Incubate for 2 h at 37°C for both enzymes. Store on ice until gel analysis. Four digests in all.



The restriction enzymes cut in the 4-base sequences shown below:

*Bsh*1236I	5'…CG|CG…3' 3'…GC|GC…5'
*Rsa*I	5'…GT|AC…3' 5'…CA|TG…5'

16S rDNA sequencing:

The 16S rDNA PCR products of the two clinical isolates are sent for sequencing.

(1D) AB test and blue plates.

Use a sterile cotton swap to pick up a couple of colonies of each of the three unknown strains. Tip: make sure you pick the colonies with side of the tip of the cotton swap not the very tip. This is important for the next step: Resuspend the picked colonies in 1 mL of sterile NaCl in an eppendorf tube. Remember to label the tube. Label each of the three BA plates which are preincubated with the Van tablet with team number and strain, ex1-1, ex1-2 and ex1-3. Spread the samples by taking a new sterile cotton swap and dip it into the eppendorf tube with the test organism. Press the cotton swap gently against the side of the tube to remove excess liquid, then spread the inoculum by wiping the surface of the BA plate while rotating the swap. Make sure that the entire sureface of the plate is covered by several passes of the swap.

After spreading place a Rosco tablet with ampicillin, gentamycin and polymyxins over the letters A, G, and P, respectively.

Use a sterile inoculation loop to transfer one colony of each of the tree strains to 1/3 of a blue plate (i.e. all 3 strains go to one plate). Take two new inoculation loops in sequence to streak out to single colonies.

Incubate all 4 plates o.n. upside down at 37C in plastic bags.

(1E) BIOLOG test.

The BIOLOG test is a classical biochemical reaction test where each strain is grown in several different carbon sources. Identification is based on a database describing thousands of different species, and a certain pattern of acceptable carbon sources can be translated into more or less precise id. Previously this test was performed using an array of dfferent media in normal test tubes and other characterizations such as motility test, gas formation tests etc., followed by manual reading and look-ups in data manuals such as Bergey's manual of Systematic Bacterology. A copy of this manual is available in our library. See also a flow chart used for id here (IDFlowcharts.pdf available on the fileshare, source: http://www.uiweb.uidaho.edu/micro_biology/250/IDFlowcharts.pdf)

Now this process has been automated and we can utilize a robot to do the work for us.

From BA plates with pure colonies (ONLY strain 1 and 2!!!) one colony is picked up with a sterile cotton swap and resuspended in a tube of IF-A (BIOLOG inoculation fluid A). The turbidity is checked using the designated BIOLOG turbidometer. Turbidity should be transmittance 90-98%. If the reading is outside this range, the tube is adjusted by adding more liquid from another tube or by adding more cells. When the %T is correct a GEN-III BIOLOG plate is inoculated using the electronic multichannel pipette with 100 µl per well.
NOTE: The BIOLOG plates cost approx.120 kr each (2011), so please be careful.

Clearly mark the plate (the bottom part, NOT the lid) on the side with team number and strain. Put the plates in the collection bin marked "BIOLOG". When all teams have inoculated their plates, a teacher will load the BIOLOG machine in room 145 and start the incubation.

Day 5 (12 in the timetable)

(1C) RFLP analysis: Visualization of fragments on agarose gel

Materials
Agarose
1xTBE buffer (for gels and electrophoresis apparatus)
EtBr
15 µL 6x Loading buffer
Sterile H_2O
λ-standard

Prepare a 1.7 % (w/v) agarose gel by dissolving 3.4 g agarose in 200 ml 1 x TBE buffer (heat until boiling and melting of the agarose in a microwave). Add 10 µl EtBr-solution before pouring the agarose solution in the gel cast, the agarose must have cooled of til aprox. 50 ºC.
Analyze the 4 digests and 2 uncut PCR products and negative control + a λ EcoRI/HinDIII standard (8 samples in all) by agarose gel electrophoresis for 2 - 3 h (~ 70 volt).
Prior to use dilute the loading buffer by mixing 37,5 µL H2O with 15 µL 6x Loading buffer.

Load with the following mixture:
5 µL DNA
7 µL diluted loading buffer

Warning – Ethidium bromide is carcinogenic! Work with gloves.

The following can be done when time allows:

(1C) Identification of sequence using blast analysis

From the sequences received from the sequencing company, the clinical isolates are identified by using a search program called Blast. This can for instance be found at PubMed.
Go to http://www.pubmed.com, then Select: "Nucleotide" in the top black bar, then select: "BLAST" on the left menu. Different search tools are found, select "nucleotide blast" (blastn) to compare a nucleotide sequence with a nucleotide database.

(1E) Identification using BIOLOG

Read the output from the BIOLOG robot and compare to the other findings from the rest of the exercise.

Supporting paper:
Amann, R. et al (1996): In situ visualization of high genetic diversity in a natural microbial community. *J. Bacteriol.* **178**(12): 3496-3500.

Direkte link: http://jb.asm.org/cgi/reprint/178/12/3496

Appendix:

Statens Serum Institut beskrivelser:

Blå plader:

Selektivt og indikativt medium til dyrkning og differentiering af gramnegative stave, fortrinsvis Enterobacteriaceae.
Anvendes til at screene for laktoseforgæring. Salmonella og Shigella arter er overvejende laktose-negative.
Udover Enterobacteriaceae vokser mange andre gramnegative stave på pladen.
Selektiviteten er god, og grampositive bakterier er for praktiske formål ikke i stand til at vokse på pladen.
Princip:
Selektiviteten skyldes et overfladeaktivt stof (detergent), der hæmmer væksten af grampositive bakterier.
Detergenten hæmmer desuden Proteus arter i at sværme.
Forgæring af laktose medfører syredannelse, hvilket får indikatoren, bromthymolblåt, til at slå om fra blå til gul.
pH justeret til 8,7 +/- 0,1. Vægt 30 g (9 cm)

Blodplader:

Generelt dyrkningsmedium.
Streptokokker af serogrupperne A, C og G vokser med klassisk, dvs. udtalt og næsten total b-hæmolyse på en blodplade.
Andre streptokokker - samt bakterier tilhørende en række forskellige slægter - kan vokse med varierende b-hæmolyse på denne plade.
Princip:
Tilsætningen af blod gør det muligt at iagttage forskellige former for hæmolyse.
pH justeret til 7,4 +/- 0,1. Vægt 20-23 g (9 cm).